Research Paper

Neuropilin-2 acts as a modulator of Sema3A-dependent glioma cell migration

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Semaphorin 3A (Sema3A) is a secreted guidance molecule initially described in the nervous system. This protein is able to control axon growth but also effects on endothelial cells migration. Here, we report that Sema3A acts as a chemorepellent factor for the rat C6 glioma cells and three different human glioma cell lines. Interestingly, Sema3A triggered a chemoattractive response in a fourth human glioma cell line. The nature of the receptor complex ensuring the appropriate signaling was dissected in C6 cells by using function blocking antibodies and gain- or loss-of-function experiments using recombinant receptors. Our results demonstrate that neuropilin-1, neuropilin-2 and PlexinA1 are necessary to trigger cell repulsion. The selective blockade of neuropilin-1 or Plexin-A1 switched the chemorepulsive effect of Sema3A into a chemoattractive one. Strikingly, blocking Neuropilin-2 suppressed Sema3A-induced cell migration while overexpression of neuropilin-2 was able to convert the chemorepulsive effect of Sema3A into a chemoattractive one. Our results not only provide additional evidence for a biological function of Sema3A in glioma migration but also reveal part of the receptor complex involved. Hence, our study describes a receptor-based plasticity in cancer cells leading to opposite migration behavior in response to the same extracellular signal.

Introduction

The semaphorins have been initially characterized as key regulators of the nervous system development.[1] These transmembrane or secreted proteins act locally or at a distance of the cells through the establishment of gradients.[2] Semaphorins are bifunctional molecules exhibiting growth promoting or growth inhibitory effects[3] thought to contribute to their complex roles in the overall cellular behavior including cell migration, differentiation or cell death.[4] The diversity of functions encoded by semaphorins is supposed to be related to the formation of specific receptor complexes including various partners such as the neuropilins (the ligand binding subunits of semaphorins),[5] members of the plexin family,[6] VEGF receptors (VEGFR1 and VEGFR2),[7] adhesion molecules (L1, NrCAM)[8] or integrins.[9] Increasing evidence is nowadays demonstrating the biological functions of various semaphorins in cancer and tumorigenesis.[10] Most of these studies focused on two members of the class 3 semaphorin (SEMA3B and SEMA3F) whose genes are located on chromosome 3, p21.3 in humans, a region frequently deleted in cancer.[11] As a result, low levels of expression of SEMA3F and SEMA3B are considered to be in favor of tumor progression especially in lung and mammary cancers. Our previous work suggested that another class 3 semaphorin, Sema3A which is probably the best documented semaphorin both in term of function and signaling, could play a role in tumor cell migration. This work showed that Sema3A induces repulsion or cell death of human medulloblastoma cells[12] through the selective recruitment of the MAP kinase ERK1/2 and p38 respectively.[13] Moreover, it has been shown that SEMA3A regulates chemotaxis of carcinoma cells by competing with autocrine pathways of VEGF.[14] Nevertheless, the role of Sema3A in cancer progression remains poorly described although Sema3A has been reported tocontrol angiogenesis, a critical step in cancer progression, by inhibiting integrin function.[15] Based on studies having shown the role of Sema3A during glial precursor migration[16] and oligodendrocyte migration[17] or differentiation,[18] we decided to investigate the possible involvement of Sema3A in the control of glial tumors. Gliomas are one of the most common tumors of the central nervous system in humans. These aggressive tumors have generally bad prognostic and lack efficient treatments. Our study was also motivated by a recent publication which showed that human glioma cells express major receptors of semaphorins but failed to provide clear functional effects of these molecules in this cellular contest.[19] Hence, Neuropilin-1, the binding receptor of Sema3A, promotes glioma progression.[20] To address the biological function of Sema3A in glioma, we performed a systematic analysis of the rat C6 and human glioma cell lines in a model of 3-dimensional migration. Coculture assays allowed us to create stable gradients of Sema3A allowing cell migration analysis. We also performed gain

and loss of function experiments in the C6 cell line to dissect part of the molecular complex involved in Sema3A-related cell migration. Our results provide evidence for a role of a receptor triad composed at least of Neuropilin-1 (NRP1) which associates with NRP2 and PlexA1 to trigger C6 cells repulsion. Moreover, we show that NRP2 is able to modulate the sensitivity of C6 cells to Sema3A by converting its chemorepulsive effect into a chemoattractive one. This result is consistent with the migration behavior of various human glioma cell lines exposed to Sema3A gradients. Our results not only provide additional evidence for a biological function of Sema3A in glioma migration but also reveal part of the receptor complex involved. Hence, our study describes a receptor-based plasticity in cancer cells leading to opposite migration behavior in response to the same extracellular signal.

Results

Lack of proliferative or cytotoxic effect of Sema3A. Sema3A has been shown to induce multiple biological effects. To address this point, we first performed cell cycle analysis to search an effect at the level of cell proliferation in response to exogenous Sema3A application. As seen in Figure 1A, the addition of 100 ng/ml purified Sema3A had no effect on C6 cell cycle after 48 h culture. Additional analysis performed at 24 hr (n = four independent experiments) and 96 hr (n = two independent experiments) provided similar results (data not shown). Moreover, consistent with the lack of sub G_0/G_1 cell fraction,
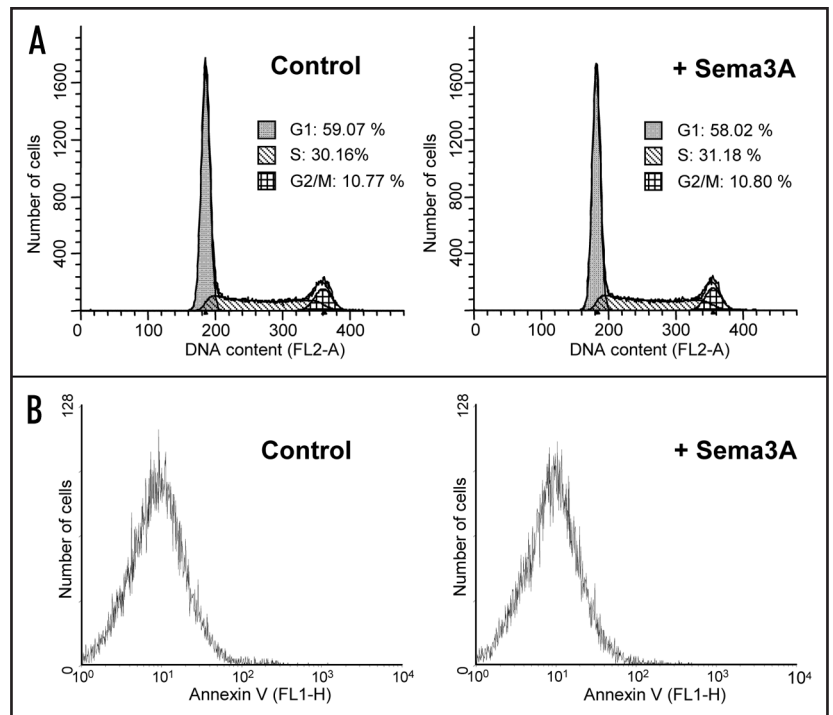

Figure 1. Determination of the proliferative and survival capacity of C6 cells in the presence of Sema3A. (A) Flow cytometry analysis was performed to determine the DNA content histogram to estimate the proliferative capacity of C6-cells. No significant difference was found in the percentage of cells in S + G_2/M phase between control and Sema3A treated C6 cells after 48 hours treatment (n = three independent experiments, the figure is showing a representative experiment). (B) Cell death in the presence of Sema3A was investigated using annexin V staining test. No increased apoptosis was found after 24 hours Sema3A-treatment (n = three independent experiments, the figure is showing a representative experiment).

the analysis of the number of Annexin-V positive cells with or without Sema3A treatment showed no induction of apoptotic cell death. Thus, Sema3A has no effect at the level of cell proliferation and cell death in C6 glioma cells.

Sema3A induces C6 glioma cell repulsion. To further evaluate the biological effect of Sema3A in glioma cells we examined cell migration using a coculture assay in plasma clot. As seen in Figure 2A, C6 cells migrated in a radial way when exposed to control aggregates of mock-transfected HEK cells (average migration index: 0.04; n = 79). In the other hand, C6 cells co cultured with Sema3A-secreting HEK cells migrated away from the semaphorin source (mean guidance index: -0.24; n = 77). Thus, our results demonstrate the existence of a repulsive effect of Sema3A on the migration of C6 glioma cells (Fig. 2A and C).

Sema3A-induced C6 cell repulsion is mediated by NRP1. To analyze the receptor complex triggering Sema3A-induced C6 cells repulsion we performed gain- and loss of-function experiments. We generated stable NRP1-overexpressing C6 cells (C6-NRP1; Fig. 2B) and repeated coculture experiments with HEK-Sema3A cells. Under these conditions we observed a significant increase (+70%) of Sema3A repulsive effect as determined by the number of aggregates showing asymmetrical negative migration index (mean guidance index: -0.41; n = 50). Moreover the role of NRP1

in mediating the repulsive effect of Sema3A was confirmed by loss-of-function experiments using a specific function blocking antibody against NRP1. As expected, the addition of the antibody abrogated the chemorepulsive effect mediated by Sema3A on C6 cells confirming the requirement of NRP1 in Sema3A-induced chemorepulsion (Fig. 2C). However, blocking the function of NRP1 switched the C6 cells behavior towards chemoattraction (Fig. 2D) in the presence of Sema3A-secreting cells (mean guidance index: +0.43; n = 25 aggregates).

Plexin-A1 is required for Sema3A-induced C6 cell repulsion. PlexA1 has been shown to transduce the Sema3A-chemorepulsive signaling in different cell types.[26] We therefore tested whether PlexA1 also mediates C6 cell repulsion when exposed to Sema3A gradients. To this end we used a dominant negative form of PlexA1 deleted in its cytoplasmic signaling domain (PlexA1$^{\Delta cyt}$). We generated a C6 cell line stably expressing PlexA1$^{\Delta cyt}$ (C6-PlexA1$^{\Delta cyt}$) and co cultured it with Sema3A-producing cells for 48 hours. The results showed that Sema3A-induced repulsion was suppressed in these C6-PlexA1$^{\Delta cyt}$ cells. Strikingly, overexpressing PlexA1$^{\Delta cyt}$ also induced a switch towards a chemoattractive effect of Sema3A in C6 cells (mean guidance index: +0.26; n = 30 aggregates). Together these results suggest that a functional NRP1/PlexA1 complex is required for Sema3A-induced chemorepulsion of C6
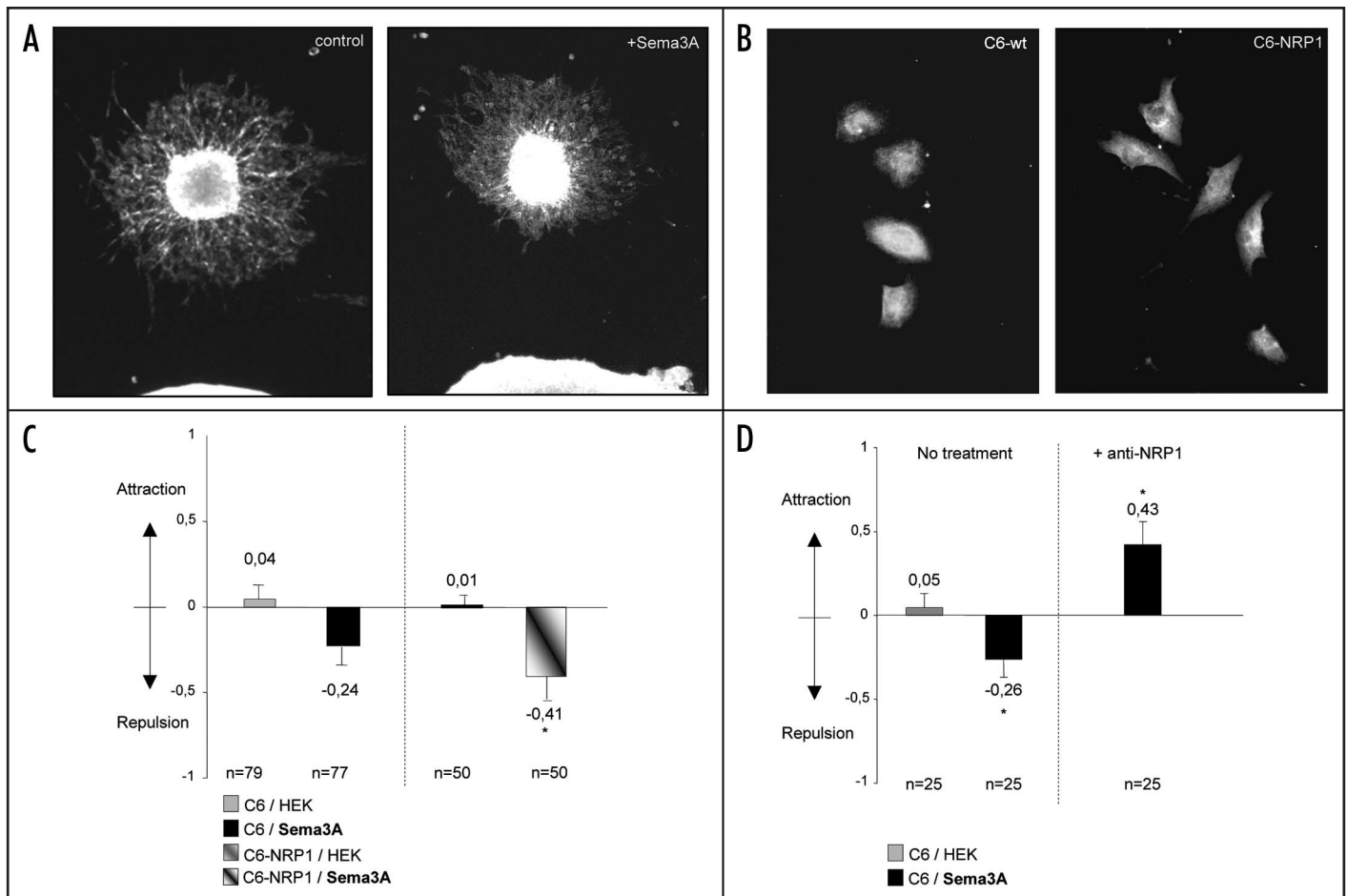
Figure 2. Sema3A induces repulsion of C6 cells. (A) Microphotographs of C6 cell aggregates illustrating the migration of C6 cells after 48 h coculture with control or Sema3A-expressing cells. Note the asymmetric outgrowth of the cell aggregate exposed to Sema3A gradient. (B) Expression of NRP1 in wild type C6 cells (C6-wt) and C6 cells overexpressing NRP1 (C6-NRP1). (C) Determination of the guidance index. Null index (0) was attributed to aggregates when cells had no preferential direction of growth while negative value (-1) was attributed to aggregates when glioma cells migrated away from the semaphorin-secreting HEK aggregate. The results are showing the average guidance index obtained in control or Sema3A conditions with C6 cells or C6 cells overexpressing NRP1. (D) Coculture experiments were also performed in the presence of a function blocking antibody against Neuropilin-1. Note that under these conditions C6 cells were attracted towards the source of Sema3A (positive guidance index). (*p < 0.05, n = number of cell aggregates, χ^2 analysis).

cells. Blocking NRP1 or PlexA1 allows the formation of a different receptor complex converting Sema3A-induced repulsion into attraction (Fig. 1).

NRP2 is necessary to trigger Sema3A-induced C6 cell repulsion and attraction. To identify the receptor component mediating C6 cells attraction, we focused on NRP2 because recent data suggest in the zebrafish that the binding of Sema3D on a homodimer of NRP1 induces repulsion while the binding of Sema3D on a heterodimer composed of NRP1 and NRP2 induces attraction.[27] We also have shown that the Sema3C-chemoattractive effect on cortical axons requires NRP2.[28] Loss of NRP2 function experiments were performed by coculturing Sema3A-producing cells in the presence of an anti-NRP2 antibody previously shown to inhibit NRP2 signaling[29,30] (Fig. 4A). Strikingly, the chemorepulsive effect of Sema3A observed in control conditions was abolished in the presence of the NRP2-blocking antibody (mean guidance index: +0.1; n = 25). Unlike the results described in

NRP1 and PlexA1 loss of function studies, the blockade of NRP2 did not converted the chemorepulsive effect of Sema3A into a chemoattractive one. This suggests that NRP2 is required for Sema3A-induced repulsion but might also mediate the functional switch from repulsion to attraction. Further insights were obtained by overexpressing NRP2 in C6 cells and determining their responsiveness to Sema3A. As seen in Figure 4B, high levels of NRP2 lead to a chemoattractive effect of Sema3A (mean guidance index: +0.42; n = 29). Thus, NRP2 is able to modulate the migration behavior of C6 cells in response to Sema3A.

Effects of Sema3A on human glioma cells. We next examined the potential effect of Sema3A on the migration of four human glioma cell lines. These cell lines were obtained from patient biopsies and were diagnosed as glioma based on anatomopathologic examination, MR imaging and aggressiveness. Although their in vitro growth was generally very slow (up to one month to reach confluence of a 10 cm large petri dish), we were able to

perform some coculture experiments and address their sensitivity to Sema3A at 48 h in culture together with analysis of NRP1, NRP2 and PlexA1 expression at the protein level (Table 1). In general all cell lines tested responded to Sema3A. Strikingly, in three of the four cell lines tested, Sema3A exerted a chemorepulsive effect on the migration of the cells (GLA8 mean guidance index -0.33, n = 20; GLA11 mean guidance index -0.6, n = 29; GLA14: mean guidance index -0.33, n = 30). Intriguingly, Sema3A triggered a chemoattractive effect on the migration of the cell line GLA7 (mean guidance index +0.6; n = 36). To understand these different effects mediated by Sema3A, we investigated the presence of the three subunits of class 3 semaphorin receptor complex, NRP1, NRP2 and PlexA1 by immunocytochemistry. Cells from GLA8, GLA11 and GLA14 expressed NRP1, but GLA7 did not express NRP1. All cell lines expressed NRP2 and PlexA1. Thus, the atypical migration behavior observed for GLA7 may be related to the lack of NRP1 expression. Overall, our results demonstrate that human glioma cell lines are sensitive to Sema3A and that the biological response is receptor-dependent (Table 1).

Discussion

Numerous studies have now demonstrated the implication of semaphorins and their receptors in tumor development and progression particularly in lung and mammary cancers.[10,31] The open questions are the generalization of such roles in the physiopathology of all cancer types and the signaling mechanisms associated. Here, we provide evidence for the existence of a chemorepulsive effect of Sema3A on the migration of the rat C6 glioma cells and various human glioma cells. Strikingly, our results demonstrate that at least C6 cells are able to modify their sensitivity to Sema3A as a function of receptor recruitment. The observed conversion of the chemorepulsive effect into a chemoattractive one appears to be in part Neuropilin-2 dependent while all three receptors NRP1, NRP2 and PlexA1 are necessary to trigger the chemorepulsive effect.

Sema3A has been initially identified in the developing nervous system as a repellent factor for various axon tracts.[1,22,32] Subsequent studies identified a role of Sema3A during angiogenesis[33] and tumor cell migration.[12,14] Here we report evidence for a chemorepulsive effect of Sema3A in glioma cell lines. Most of our work was performed on the rat C6 glioma cell line which represents a good model of invasive tumor.[34] Interestingly, Sema3A did not induced neither cell death of C6 cells as previously described for sympathetic neurons[35] or medulloblastoma cells[12] nor inhibition of cell proliferation as previously obtained with SEMA3B in breast carcinoma[36] and SEMA3F in MCF7 cells.[30] Such functional difference illustrates cell type-specific biological effects for a given semaphorin. Gain of function experiments in which a recombinant NRP1 was overexpressed in C6 cells confirmed the relationship between the occurrence of cell repulsion and the level of NRP1 expression. In accordance to the literature,[37] we also found that the repulsive effect of Sema3A required PlexA1 as shown in function blocking experiments using a PlexA1 mutant with cytoplasmic domain deletion.[38] This member of the type-A plexin family is largely considered to be the transduction subunit of Sema3A signaling by interacting with
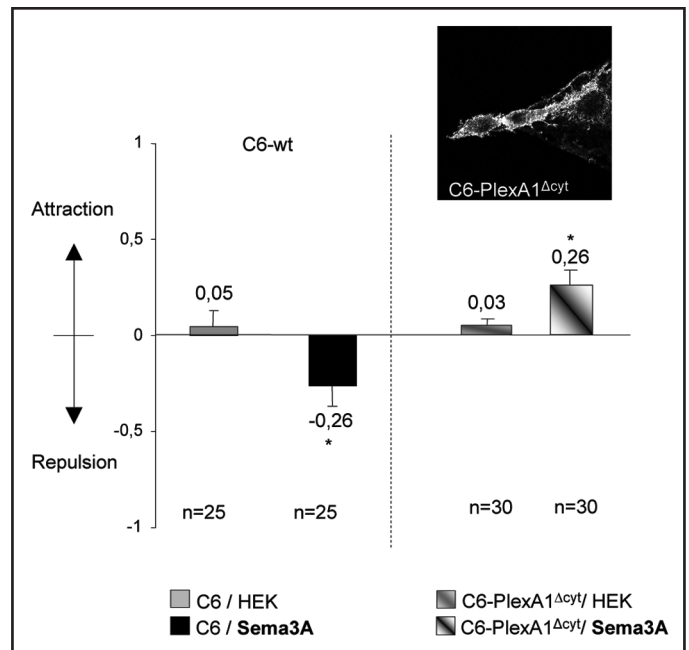

Figure 3. Plexin-A1 is required to induce the C6 cell repulsion mediated by Sema3A. C6 cells overexpressing a truncated form of PlexA1 subunit (immunostaining of PlexA1$^{\Delta cyt}$ using the anti-VSV tag of the recombinant protein) were cocultured with control or Sema3A-expressing cells. Compared to wild type C6, C6 cells expressing PlexA1$^{\Delta cyt}$ were attracted by a gradient of Sema3A. (*p < 0.05, n = number of cell aggregates, χ^2 analysis).

Table 1 **Expression of NRP1, NRP2 and PlexA1 receptors and biological effects of Sema3A in 4 human glioma cell lines at 48 h coculture**

Human glioma	NRP1 expression	NRP2 expression	PlexA1 expression	Effects of Sema3A
GLA7	-	+	+	Attraction GI = +0.6
GLA8	+	+	+	Repulsion GI = -0.33
GLA11	+	+	+	Repulsion GI = -0.6
GLA14	+	+	+	Repulsion GI = -0.33

A chemorepulsive effect was found for GLA8, GLA11 and GLA14 expressing NRP1, NRP2 and PlexA1. A chemoattractive effect was found for GLA7 expressing NRP2 and PlexA1. GI, guidance index.

GTPases of the Rho family.[25,39,40] Interestingly, the inactivation of NRP1 or PlexA1 not only suppressed the inhibitory effect of Sema3A but converted it into a chemoattractive one. Strikingly, the analysis of the biological function of NRP2 strongly suggests that this functional switch is NRP2 dependent. As a matter of fact, the conversion was not observed in the case of NRP2 function blocking experiments while overexpressing of NRP2 in C6 cells triggered a chemoattractive effect of Sema3A. Such functional switches have already been described in the nervous system for the semaphorin receptor L1 which is able to convert the chemorepulsive effect of Sema3A into a chemoattractive one.[41] The recruitment of NRP2 has already been proposed to trigger the Sema3D-induced attraction by the formation of an heterodimer with NRP1.[27] Our
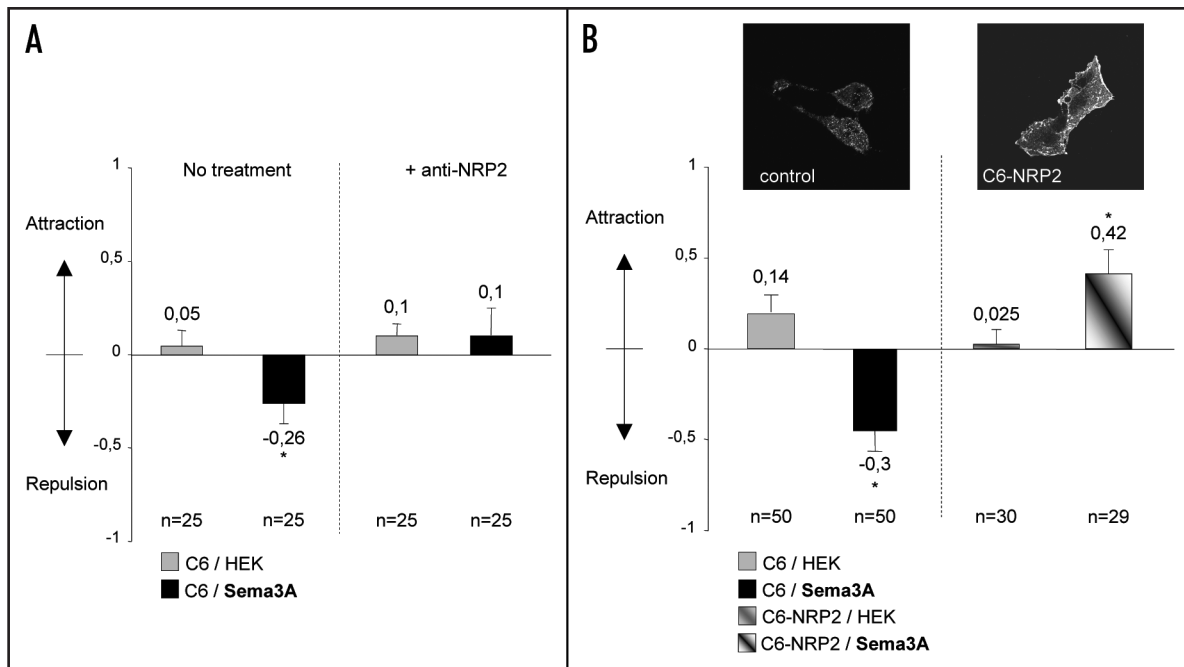
Figure 4. NRP2 is required to induce C6 cell repulsion and attraction. (A) Coculture assay in presence of a function blocking antibody against NRP2. The repulsive effect of Sema3A is abolished when NRP2 is blocked. (B) Gain of function experiment in coculture assay with C6 cells overexpressing NRP2 (C6-NRP2). The microphotographs illustrate the relative amount of NRP2 in wild type cells and in C6-NRP2 cells. Overexpression of NRP2 switched the chemorepulsive effect of Sema3A into a chemoattractive effect. (*p < 0.05, n = number of cell aggregates, χ^2 analysis).

results provide evidence that the relative amount of NRP2 is able to modulate cell sensitivity to Sema3A. The existence of a NRP1/NRP2 heterodimer has been suggested.[42] However, the binding of Sema3A to NRP2 remains controversial. Our own data (see Suppl. Fig. 1) suggest that Sema3A binds NRP2 but 1.3 fold less that NRP1. The identification of the receptor component signaling the growth promoting effect of NRP2 will be difficult to achieve taking into account the diversity of the coreceptors nowadays describes for NRPs signaling.[43,44] Moreover, we cannot exclude the existence of a direct binding of Sema3A to a transducing receptor as previously described for Sema3E interaction with PlexD1.[45] Thus, the exact nature of the receptor complex enabling Sema3A to exert a positive growth promoting effect remains to be elucidated. Nevertheless, our results obtained with multiple receptor blockades suggest that the chemorepulsive effect of Sema3A in C6 cells requires the participation of NRP1, NRP2 and PlexA1 to be fully functional. In this context, NRP2 appears as a modulator of cell signaling by its capacity to convert the repulsive effect into an attractive response.

Here, we also investigated how human glioma cell lines respond to Sema3A. We found that three of the four glioma cell lines obtained from patient biopsies expressed NRP1, NRP2 and PlexA1 similarly to what was described for some of human cell lines described by Rieger and collaborators.[19] In this study the authors failed to demonstrate a functional role of Sema3A in coculture experiments. This apparent discrepancy may reflect some differences in the culture method (collagen gel versus plasma clot) but more presumably resides in the lack of PlexA1 expression in the U87MG cell line tested by Rieger and colleagues. In our

experiments, all the human glioma cell lines repelled by Sema3A expressed the triad NRP1/NRP2/PlexA1. The migration behavior of GLA7 cells, which was attracted by gradients of Sema3A, is particularly interesting because these cells did not express NRP1. It is tempting to speculate that this growth promoting effect is evoked by a NRP2-dependent signaling pathways similar to the one observed in C6 cells overexpressing NRP2. Future studies will investigate how interference of the receptor complex of semaphorins must be done to obtain a therapeutic benefit. This will be done by taking into account the expression levels of semaphorins and their receptors by tumor cells themselves. Indeed, C6 cells express Sema3A (see Suppl. Fig. 2). As recently described for other glioma cell lines, this may reflect the existence of autocrine functions of Semaphorins influencing tumor cell dispersal.[46] This mechanism is extremely important considering semaphorins as versatile regulators of tumor progression and tumor angiogenesis.[47]

Materials and Methods

Cell lines. Rat C6 glioma cells (ATCC CCL-107) were grown in MEM with 5,000 u/ml penicillin, 5 mg/ml streptomycin, 200 mM L-glutamine and 10% fetal calf serum.

Stably transfected human embryonic kidney 293 cells (HEK293 cells, ATCC CRL-1573) expressing functional recombinant Sema3A (pBKFlagSema3AP1b)[21-23] or mock-transfected (control) cells were cultured in minimal essential medium containing 5,000 u/ml penicillin, 5 mg/ml streptomycin, 200 mM L-glutamine, 10% fetal calf serum and 1 mg/ml G418 (Life Technologies).

We also used 4 human glioma cell lines (GLA7; GLA8; GLA11; GLA14) established in our laboratory in collaboration with Pr P.

Kehrli (Hautepierre Hospital, Strasbourg) from post-operating tumors and cultured in DMEM supplemented with 10% fetal calf serum.

Coculture assay. Cell aggregate were produced as previously described.[22] In brief, cells were trypsinized before suspension in 2 ml of primary culture medium. Drops of the cell suspension (20 μl) were placed onto the lids of 35 mm dishes, which were inverted and incubated over culture medium for 24 hours. Each cell cluster was harvested into medium and trimmed in four pieces with tungsten needles. Cell aggregates were placed in 10 μl chicken plasma (Sigma) on a glass coverslip. During coagulation of the clot with 10 μl thrombin (Sigma), tumor cell aggregates were arranged around semaphorin-producing HEK cells aggregates at 500–1,000 μm distances. After 20 minutes, coverslips were placed in 35 mm dishes containing 2 ml culture medium and transferred to the incubator (37°C, 5% CO_2) for 24–48 hours. Blocking experiments were performed with C6 cells by incubating aggregates with 1 μg/ml anti-NRP1 polyclonal antibody obtained using a synthetic 14 amino acid peptide from the MAM domain of NRP1;[12] or with 1 μg/ml anti-NRP2 polyclonal antibody (Santa Cruz Biotechnology; Santa Cruz CA). Control experiments were performed to exclude a possible cross reactivity between anti-NRP1 and anti-NRP2 (see Suppl. data). These two anti-neuropilin function blocking antibodies have been used previously used by several groups. To inactivate PlexA1 we performed experiments with C6 cells stably expressing a dominant negative form of PlexA1 (PlexA1-Δcyt lacking intracellular domain, gift from AW. Püschel). Stable cell lines overexpressing NRP1 and NRP2 were also used in gain of function experiments. After 48 h, cultures were fixed in 4% formaldehyde and analyzed using phase-contrast optics (Zeiss, Jena, Germany). Guidance indexes were determined in the different conditions as a function of the general growth of tumor cells towards (+1) or away (-1) from the central aggregate. Statistical significance between the different mean values was determined using the chi square test. A minimum of three independent experiments were performed by independent researchers placed in blind conditions for analysis. Results are presented as mean +/- standard errors, n indicate the number of cell aggregates for a given condition.

Flow cytometry. For the proliferation and cell death assay, the cells were incubated at a known density in 60 mm dishes (falcon) (100 x 10^3 cells for 24 and 48 hours treatment and 20 x 10^3 cells for 96 hours follow up). After 24 h, half of the medium was removed and replaced with control or Sema3A enriched conditioned medium. Cells were left for 24, 48 or 96 hours treatment in the incubator (37°C, 5% CO_2).

For cell cycle analysis, cells were collected and fixed with 70% ethanol at -20°C overnight. The DNA content was revealed using a staining buffer containing 25 μg/ml propidium iodide and 50 U/ml RNAse A (Sigma) in PBS. The cell cycle status was analyzed using a flow cytometer (FacsCalibur, Becton Dickinson) and cell cycle analysis software (Modfit LT). Apoptotic cells were detected by staining the samples with Annexin V-FITC (Becton Dickinson, Annexin V-FITC apoptosis detection kit I). The obtained FACS-histograms were analyzed using WinMDI software.

Immunocytochemistry. Coverslips were prepared with 2 x 10^5 C6 cells. Cells were fixed in 4% formaldehyde for 10 minutes and then incubated in PBS with 3% BSA for 15 minutes. A first antibody was applied for 2 hours at room temperature: NRP1 rabbit polyclonal antibody (1:100; Oncogene, Darmstadt, Germany) generated by immunizing rabbits with a synthetic peptide corresponding to amino acids 813–827 of the rat neuropilin-1 protein, or NRP2 rabbit polyclonal antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) raised against a recombinant peptide corresponding to amino acids 560–858 mapping within the extracellular domain of NRP2 of human origin, or Plexin-A1 Nter rabbit polyclonal antibody (1:500) raised against a synthetic peptide corresponding to amino acids 1–21 mapping within the NH2-terminal domain of Plexin-A1 of human origin.[24] A goat anti-rabbit antibody bound to CY3 (1:1,000; Interchim, Montluçon, France) was used as secondary antibody. Coverslips were mounted in Aqua-polymount (Bioscience Inc., Washington) before microscopic analysis using a Zeiss microscope equipped for RITC fluorescence. The expression of NRP1, NRP2 and PlexA1 in human glioma cell lines was performed using the same protocol.

Overexpression of full length and truncated Sema3A receptors. NRP1, NRP2 and Plexin-A1$^{\Delta cyt}$ cDNAs have been cloned in pBK-CMV (Stratagene) as previously described.[25] The transfection procedure was performed using 10^6 C6 cells. Lipofectamine™ 2000 (1 mg/ml) was mixed gently before use, and 90 μl was diluted with 0.9 ml of Opti-MEM I medium. The solution was incubated for 5 minutes at room temperature before addition of DNA (9 μg). After 30 minutes incubation at room temperature, 7.2 ml of Opti-MEM I medium was added. The cells were rinsed with Opti-MEM I Medium, and the solution containing DNA, Lipofectamine™ 2000 and Opti-MEM I medium was placed directly on the C6 cells. The cells were incubated at 37°C in a CO_2 incubator for 6–24 hours. The transfection solution was removed and replaced by a medium containing Serum and G418 to select transfected cells. Expression level of overexpressed receptors was checked by western blot (data not shown) and immunocytochemical analysis.

Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/NasarreCAM3-4-Sup.pdf

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